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MOLECULAR GENETIC CHARACTERIZATION OF PATHOGENICITY ISLANDS SPI-II AND SPI-III OF *Salmonella* STRAINS ASSOCIATED WITH SEAFOODS

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MOLECULAR GENETIC CHARACTERIZATION OF PATHOGENICITY ISLANDS SPI-II AND SPI-III OF *Salmonella* STRAINS ASSOCIATED WITH SEAFOODS

Salmonella are food/water borne Gram-negative, rods belonging to the family *Enterobacteriaceae* which cause clinical conditions ranging from mild gastrointestinal infection to systemic infections such as typhoid fever. Though there are only two species of *Salmonella viz. S. enterica* and *S. bongori*, it consists of more than 4500 serotypes. Among the diseases caused by *Salmonella*, nontyphoidal *Salmonella* (NTS) infections have significant effects on human health which are reported to be mainly associated with consumption of contaminated food and water. Understanding the human health significance of seafood associated *Salmonella* is important for the assessment of risk to human health. In the past phenotypic characteristics including serotyping have been used for epidemiological investigation of *Salmonella*. However, serotyping lacks the ability to differentiate various isolates belonging to the same serotype. Therefore a reliable molecular based discriminatory methods such as the Random amplified polymorphic DNA-PCR, the enterobacterial repetitive intergenic consensus sequences based PCR (ERIC-PCR), ribotyping, Pulse field gel electrophoresis etc. are proving to be highly usefull.

A number of virulence genes have been identified which are mainly responsible for pathogenesis of *Salmonella* and located in clusters called *Salmonella* Pathogenicity Islands (SPI). There are 17 Pathogenicity Islands of *Salmonella* reported to date. During early stage of infection the bacteria adhere to the apical epithelial surface using various fmbrial adhesins and then disrupt the normal epithelial brush to enhance the membrane ruffling for engulfing itself using a distinct type three secretion system (T3SS) which is coded by *Salmonella* Pathogenicity Island-1 (SPI-1). After invasion *Salmonella* cross the lymphoid cells and upon encounter with submucosal macrophages form the *Salmonella*-containing vacuole (SCV) to survive and multiply inside the host using a unique T3SS encoded by *Salmonella* Pathogenicity Island 2 (SPI-2). *Salmonella* possess another Pathogenicity Island, SPI-3 which help it growth in low Mg^{2+} conditions. However, the role of the virulence genes in nontyphoidal serotype which are isolated from the environment has not been studied in detail. Therefore screening for deletion of virulence genes may help to explain the reason for stringent host adaptation of seafood associated serovar of *Salmonella*. Study of the mere presence of genes may not reveal sufficient the differences between isolates in relation to

the virulence. Investigation of the expression of virulence gene is of utmost importance. Generally, *Salmonella* spend a good part of their lives as residents in animal hosts. The host environment provides *Salmonella* a warm constant temperature, as well as high concentration of free amino acids and sugar, which are favorable to bacterial growth. Once excreted from an animal host, *Salmonella* find themselves battling for survival, facing limited nutrient availability, osmotic stress, large variations in temperature and pH, and predation. To survive such stressful conditions, *Salmonella* resort to different strategies *viz*, entering a viable but nonculturable (VBNC) state, production of biofilm and using *Acanthamoeba* as environmental reservoir. Therefore, it is necessary to lock into the survival ability of *Salmonella* inside the host (HeLa cells and *Acanthamoeba*) and the environment (biofilm) and determine the role of genes in the survival of seafood associated *Salmonella*.

With this back ground the present study was undertaken with the following objectives:

1. To study the genomic diversity of different seafood isolates of *Salmonella* using molecular techniques and phage typing.
2. To study the genes associated with pathogenicity islands SPI-2 and SPI-3 for detection of deletion of any specific genes within the pathogenicity islands of seafood associated *Salmonella*.
3. To study the intracellular behaviour of seafood associated *Salmonella* in human epithelial cells (HeLa) and expression of genes to determine the potential role of these genes in infections.
4. To understand the genes involved in environmental survival and persistence in these seafood associated strains.

The thesis is presented in the chapters as follows:

Chapter one introduces the significance of the study.

Chapter two provides an up to date review of the literature concerning *Salmonella* in seafood, molecular characterization of *Salmonella*, pathogenesis of the organism including various virulence factors and Pathogenicity Islands, and survival strategy of *Salmonella* inside the host and in environment.

Chapter three includes studies on serotyped and genetic characterization of seafood associated *Salmonella* isolates using DNA based fingerprinting (RAPD and ERIC) and whole cell SDS-PAGE profiling to present the genetic diversity of seafood associated *Salmonella*. Fifty eight *Salmonella* isolates obtained from seafood (21 isolates from oyster, 16 isolates from clam, 15 isolates from fish and 6 isolates from shrimp) were used in this study. The isolates were confirmed as *Salmonella* as they were positive for *hns* and *invA* gene giving amplicons of 152 and 284 bp respectively. RAPD and ERIC-PCR result elucidate the genetic difference within *Salmonella* serovars associated with seafood. The DI value obtained by RAPD and ERIC-PCR was 0.94 and 0.96, respectively (>0.90) which is the acceptable confidence value for interpreting the level of discrimination (Khoodoo *et al*., 2002; Kumar *et al*., 2008b). RAPD with 13 and ERIC with 17 different patterns could differentiate the seafood isolates indicating the prevalence of diverse *Salmonella* serotypes in this region. Along with RAPD and ERIC-PCR, Whole cell protein profiles of 58 *Salmonella* isolates on SDS-PAGE were carried out for epidemiological investigation of *Salmonella*. Result showed major bands of ~ 38 kDa and ~ 46 kDa among all the isolates. Present study shows that, use of protein profiling in combination with established typing methods such as RAPD and ERIC-PCR could provide useful information in typing of nontyphoidal *Salmonella* isolates associated with seafood to develop strategies to protect public health from *Salmonella* infections.

Chapter four presents work on screening isolates belonging to different serovars obtained from seafood for the detection of deletion of some specific genes known to be associated with SPI-2 using PCR and dot blot technique. Sixteen genes (*ssaU*, *ssaB*, *ssaR*, *ssaQ*, *ssaO*, *ssaS*, *ssaP*, *ssaT*, *sscB*, *sseF*, *sseG*, *sseE*, *sseD*, *sseC*, *ssaD* and *sscA*) of SPI-2 were targeted for PCR amplification in 57 seafood associated serovars of *Salmonella*. *sseC* gene of SPI-2 was found to be absent in two isolates of *Salmonella enterica* serovar Weltevreden, SW13 and SW39. Absence of *sseC* was confirmed by sequencing using flanking primers. SW13 had only 66 bp sequence of the *sseC* gene and SW39 had 58 bp sequence of the *sseC* gene. A clinical isolate *S.* Weltevreden3, 10:r:z6 was used to construct deletion mutant for *sseC* gene. Significant reduction in the survival of SW3, 10:r:z6 Δ*sseC* and natural mutants SW13 and SW39 in HeLa cells suggests that *sseC* has a crucial role in the intracellular survival of *S.* Weltevreden. Expression of *sseC* was upregulated during intracellular phase of both *S. enterica* serovar Typhimurium and

clinical isolate of *S*. Weltevreden suggesting crucial role for this gene in survival of *S*. Weltevreden inside host.

Chapter five describes screening of 57 seafood associated *Salmonella* isolates for the detection of four SPI-3 coded genes (*mgtB*, *mgtC*, *rhuM* and *cigR*) using PCR and dot blot technique. *rhuM* gene was found to be absent in one *S*. Weltevreden isolate (SW5) and it was confirmed by PCR using flanking primer. Amplification of *S*. Typhimurium (ST14028) genomic DNA with flanking primer pairs showed band of 1859 bp. where as SW5 showed a band of 483 bp. Dot blot analysis result showed no visual precipitation devolopment of SW5 on the nylon membrane for *rhuM* gene. In addition intracellular survival ability of SW5 using HeLa cells as cell culture model was studied. The study included quantification of the level of expression of *rhuM* gene. Significant reduction in the survival of SW5 in HeLa cells and high expression of *rhuM* gene during intracellular phase of both *S*. Typhimurium and *S*. Weltevreden suggested that *rhuM* gene might play an important role in survival of seafood.

In **Chapter six,** a sensitive and rapid multiplex PCR developed for detection of *Salmonella* Pathogenicity Island coding virulence genes in seafood associated *Salmonella* serotypes is described. This study was aimed at detecting the prevalence of 12 different virulence associated genes coded by 4 pathogenicity islands, namely, SPI-1, SPI-2, SPI-3 and SPI-5, among 31 *Salmonella enterica* spp. Two sets of Hexaplex PCR were developed. Result showed that, *hilA*, *invA*, *ssaB*, *ssaP*, *ssaQ*, *ssaT*, *sseD* and *mgtC* were 100% prevalent in this study followed by *rhuM* (96.77%), *sseC* (93.5%) and *sopB* and *pipC* (90.34%). The detection limit of the optimized multiplex PCR was tested with lowest detectable concentration of 0.005 ng μl^{-1}. This assay is a reproducible and sensitive assay for the detection of *Salmonella* by screening several virulence genes presence in SPI. Finding of presence or absence of virulence genes may explain the reason behind stringent host adaptation of seafood associated serovar of *Salmonella*.

Chapter seven describes study on the adhesion and intracellular survival of seafood associated *Salmonella enterica* serovar Weltevreden, Newport, and Bareilly along with *S*. Typhimurium (ST14028) was studied in HeLa cells. Results suggest that though seafood associated *Salmonella* serovars (*S*. Weltevreden, *S*. Bareilly, *S*. Virchow and *S*. Newport) are able to adhere and invade HeLa cells, their intracellular replication was marginal. There was a significant difference in

adhesion and survival between seafood associated *Salmonella* serotypes and *S*. Typhimurium ST14028. In addition expression level of SPI-1 and SPI-2 coded genes of different *Salmonella* serotypes after infecting with human epithelial cells was quantified using real-time PCR. Result showed that the SPI-1 coded *hilA* and *invA* genes were upregulated in all *Salmonella* with values of *S*. Typhimurium being higher than seafood associated *Salmonella*. There was no significant expression of SPI-2 coded *sseF* and *sseC* genes at 2 h and 6 h. Our results suggest that though seafood associated *Salmonella* adhere and invade epithelial cells, poor expression level of SPI-2 encoded genes may explain their inability to multiply in these cells.

In **Chapter eight** investigation of the biofilm forming ability of seafood isolates of *Salmonella enterica* serovar Weltevreden (*S*. Weltevreden) under varying nutrient conditions has been conducted. The role of cellulose production in biofilm formation and in the expression of multicellular behavior (rough, dark, red morphotype: rdar) was investigated. Fourteen isolates of seafood associated *S*. Weltevreden were studied for biofilm production in polystyrene microtitre plates. Only one (SW49) of 14 was a strong biofilm former on polystyrene template and was able to produce biofilm in both undiluted trypticase soy broth (TSB) and 1:100 diluted TSB at 24 h. All others produced moderate or weak biofilms which was better in 1:100 diluted TSB compared to undiluted medium. All the isolates except one were positive by PCR for the three genes, *gcpA* (*stm1987*), *adrA* (*yaiC*) and *csgD*. Gene expression of *gcpA*, *adrA* and *csgD* was studied by real-time PCR with the one strong (SW49) and one representative weak (SW30) biofilm former. *gcpA* was highly expressed at 24 h and 72 h in biofilm cells in both TSB and diluted (1:100) TSB. Deletion of *gcpA* in SW49 resulted in its inability to produce cellulose and consequent inability to bind calcoflour, inability to form rdar colony on Congo Red-agar plates and failure to produce biofilm on polystyrene substrate. The findings indicate that, in *S*. Weltevreden, *gcpA* is critical for activating cellulose synthesis and biofilm formation both in undiluted and diluted TSB.

Another study was aimed to observe survival ability of seafood associated *Salmonella* inside the *acanthamoeba*. Three seafood associated isolates, *S.* Weltevreden, Newport, and Bareilly along with *S*. Typhimurium (ST14028) was studied using *Acanthamoeba castellani*. Result of this study points out a symbiotic relation between *Salmonella* and *Acanthamoeba*. In addition expression level of SPI-2 coded *sseF* and *sseC* gene was quantified. Result suggested that

expression of *sseF* and *sseC* genes does not play an important role in survival of seafood associated *Salmonella* inside the amoeba which indicates seafood associated *Salmonella* is involved in a symbiotic association with *Acanthamoeba* using an SPI-2 independent pathway which yet to be investigated.

Chapter nine presents an overview of the entire research conducted. A general discussion of the results together with further studies that need to be undertaken is presented. Also included is the bibliography, summary of the work done to enable a reader to have a cursory glance of the results of this study, appendix including details of media, reagents, buffers used in the study and finally copy of peer reviewed publications, abstracts and gene bank submissions.

Published/ Accepted/ Communicated articles in peer reviewed journals

1. **Bhowmick, P.P.,** Devananda, D., Ruwandeepika, H.A.D., Karunasagar, I. and Karunasagar, I. **(2011)** Presence of *Salmonella* Pathogenicity Island 2 genes in seafood associated *Salmonella* serovars and the role of s*seC* gene in survival of *Salmonella enterica* serovar Weltevreden in epithelial cells. Microbiology **157:** 160–168. (IF: 3.025)

2. **Bhowmick, P.P.,** Devananda, D., Ruwandeepika, H.A.D., Thilo M. Fuchs., Shabarinath S., Karunasagar, I. and Karunasagar, I. **(2010)** *gcpA* (*stm1987*) is critical for cellulose production and biofilm formation on polystyrene surface by *Salmonella enterica* serovar Weltevreden in both high and low nutrient medium. Microbial pathogenesis **(In press)** doi:10.1016/ j.micpath.2010.12.002. (IF: 2.324)

3. **Bhowmick, P.P.,** Shabarinath, S., Devananda, D., Shekar, M., Ruwandeepika, H.A.D., Karunasagar, I. and Karunasagar, I. (2011) Serotyping and molecular characterization for study of genetic diversity among seafood associated nontyphoidal *Salmonella* serovars. Indian Journal of Medical Research. **(In press)** (IF: 1.82)

4. **Bhowmick, P.P.,** Devananda, D., and Karunasagar, I. **(2010)** Virulotyping of seafood associated *Salmonella enterica* subsp. *enterica* isolated from South-West cost of India. Biotechnology, Bioinformatics and Bioengineering **(Accepted).**

5. **Bhowmick, P.P.,** Devananda, D., Ruwandeepika, H.A.D., Karunasagar, I. and Karunasagar, I. Intracellular expression of genes associated with SPI-1 and SPI-2 in some seafood associated *salmonella* serovars. Microbiology **(Communicated).**

6. **Bhowmick, P.P.,** Devananda, D., Ruwandeepika, H.A.D., Karunasagar, I. and Karunasagar, I. Presence of *Salmonella* Pathogenicity Island 3 in seafood associated *Salmonella* serovars and role of *rhuM* gene in infection with human epithelial cells. **(Communicated)**

Poster Presentations (Extended abstract/ abstract)

1. **Bhowmick, P.P.,** Devananda, D., Shabarinath. S., Karunasagar, I. and Karunasagar, I. (2011) Survival of seafood associated nontyphoidal *Salmonella* inside the human epithelial cells and in the Acanthamoeba. **8th Symposium on Diseases in Asian Aquaculture** held at College of Fisheries, Mangalore, India. During 21st -25th November, 2011 (**Abstract**).

2. **Bhowmick, P.P.,** Devananda, D., Shabarinath. S., Karunasagar, I. and Karunasagar, I. (2011) Seafood associated Nontyphoidal *Salmonella* possibly has a symbiotic association with *Acanthamoeba* using SPI-2 independent pathway. **52nd annual conference of Association of Microbiologists of India** held at Panjab Univerisity, Chandigarh-160014. During 3rd-6th November, 2011 (**Abstract**).

3. **Bhowmick, P.P.,** Devananda, D., H.A Darshanee Ruwandeepika and Karunasagar, I. The study of presence of *salmonella* pathogenicity Island-3 in seafood associated *salmonella* serovars and role of *rhum* gene in infection with human epithelial cells. **51st annual conference of Association of Microbiologists of India** held at Birla Institure of Technology, Mesra, Ranchi-835215, Jharkhand, India. During 14th -17th December, 2010 (**Abstract**).

4. **Bhowmick, P.P.,** Shabarinath. S., Devananda, D., Karunasagar, I. and Karunasagar, I. Adhesion and survival of some seafood associated strains of *Salmonella* in Human epithelial cells. **50th annual conference of Association of Microbiologists of India** held at National Chemical Laboratory, Pune during 15th -18th December, 2009 (**Abstract**).

5. **Bhowmick, P.P.,** Shabarinath, S., Karunasagar, I. and Karunasagar, I. Molecular characterization of biofilm formation of seafood associated *Salmonella* Weltevreden. **48th annual conference of Association of Microbiologists of India** held at Indian Institute of Technology Madras, Chennai-600036 during 18th to 21st December, 2007 (**Abstract**).

6. **Bhowmick, P.P.,** Mallesh and karunasagar, I. Molecular characterization of seafood associated *Salmonella* involved in biofilm formation. National seminar on **"Indian Marine Fisheries Sustainability at Crossroads"** held on 22-23rd December, 2009. Organized by College of Fisheries, Mangalore (**Abstract**).

Sequnces submitted to NCBI gene bank

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Signature of the Research Supervisor **Signature of the Candidate**